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(54) LIPASES FROM HYPHOZYMA

LIPASEN AUS HYPHOZYMA

LIPASES OBTENUES A PARTIR DE HYPHOZYMA

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- **CHEMICAL ABSTRACTS, Volume 95, No. 21, 23 November 1981 (23.11.81), (Columbus, Ohio, USA), DE HOOG G.S. et al., "Hyphozyma, a New Genus of Yeast-Like Hyphomycetes", page 341, the Abstract No. 200224z, Antonie van Leeuwenhoek 1981, 47 (4), 339-352.**
- **PATENT ABSTRACTS OF JAPAN, Vol. 13, No. 549, C-662; & JP,A,01 225 481 (KURITA WATER IND LTD et al.), 8 Sept 1989 (08.09.89).**

EP 0 648 263 B1

Description

TECHNICAL FIELD

[0001] The present invention relates to novel microorganisms and to novel enzymes obtainable therefrom. More specifically, the invention relates to a new species of the genus Hyphozyma, and to novel lipases obtainable therefrom.

[0002] The invention also relates to a process for obtaining the enzymes, immobilized lipase preparations, and industrial applications of these enzymes in the paper pulp industry, for use in ester hydrolysis, ester synthesis or interesterification, and for manufacture of leather.

BACKGROUND ART

[0003] When resinous wood species are used in pulping processes, particularly mechanical pulping processes, pitch problems arise. This widespread phenomenon causes production interruptions and a decreased paper product quality.

[0004] Pitch contains considerable amounts of triglycerides, more commonly known as fats, and other esters. Fatty acid glyceride hydrolysing enzymes, in the following called lipases, may advantageously be used for efficient hydrolysis of water-insoluble esters, particularly triglycerides.

[0005] In order to comply with the prerequisite for paper pulp processing, lipases applied in methods for enzymatic pitch control should be acidophilic and thermophilic.

[0006] Enzymes suggested in the prior art for pitch control include lipases derived from strains of Pseudomonas, Humicola, Candida, Chromobacter and Aspergillus.

[0007] Some of these lipases are markedly thermophilic, others are markedly acidophilic, but none of these lipases possess both characteristics.

[0008] Hyphozyma is a new genus of yeast-like Hyphomycetes (vide de Hoog, G.S & Smith, M.Th. ; Antonie van Leeuwenhoek 47 (1981) 339-352), and the following species are reported: H. variabilis, H. variabilis var. odora, H. sanguinea, and H. roseoniger. However, no lipase production has previously been ascribed to these organisms.

SUMMARY OF THE INVENTION

[0009] We have now found that a new species of Hyphozyma is able to produce lipase. Moreover, we have found that these novel lipases possess excellent paper pulp processing possibilities due to their markedly thermophilic and acidophilic characteristics. Moreover, we have also found that these novel lipases are well suited for use in ester hydrolysis, ester synthesis or interesterification, and various other industrial applications.

[0010] In its first aspect, the present invention provides a lipolytic enzyme comprising the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Xaa Xaa Xaa Xaa Xaa Ser* Gln Gly Gly; in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

[0011] In a more specific embodiment of this aspect, the invention provides a lipolytic enzyme comprising the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Xaa Xaa Ser* Gln Gly Gly; in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

[0012] In a further specific embodiment of this aspect, the invention provides a lipolytic enzyme comprising the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Thr Xaa Ser* Gln Gly Gly; in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

[0013] In a further specific embodiment of this aspect, the invention provides a lipolytic enzyme comprising the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Pro Xcc Xcc Thr Xaa Ser* Gln Gly Gly; in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, Xbb represents a deletion or any of the naturally occurring amino acids except Asn, and Xcc represents a hydrophobic amino acid.

[0014] In a further specific embodiment of this aspect, the invention provides a lipolytic enzyme comprising the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Pro Val Leu Thr Xaa Ser* Gln Gly Gly; in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

[0015] In a further specific embodiment of this aspect, the invention provides a lipolytic enzyme comprising the following partial amino acid sequence: Gly Ser Gly Ser Xbb Lys Val Pro Val Leu Thr Xaa Ser* Gln Gly Gly; in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

[0016] In a further specific embodiment of this aspect, the invention provides a lipolytic enzyme having the amino acid sequence disclosed as SEQ ID NO 3 of the attached amino acid sequence listing, or a sequence homologue thereto.

[0017] In its second aspect, the present invention provides a process for obtaining a lipolytic enzyme of the invention, which process comprises cultivation of a lipase producing strain of the genus Hyphozyma in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the lipolytic enzyme.

[0018] In its third aspect, the present invention relates to the use of a lipolytic enzyme of the invention in the paper pulp industry for enzymatic pitch control.

[0019] In its fourth aspect, the present invention provides an immobilized lipase preparation obtained by immobilization of the lipolytic enzyme of the invention.

BRIEF DESCRIPTION OF DRAWINGS

[0020] The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows the temperature activity (% relative) at pH 6.0 of a lipolytic enzyme of the invention;
Fig. 2 shows the pH activity (% relative) at 70°C of a lipolytic enzyme of the invention;
Fig. 3 shows the relation between temperature and the degree of hydrolysis when employing the lipolytic enzyme of the invention to paper pulp (○ determined at pH 6.5; ● determined at pH 4.5);
Fig. 4 shows the relation between pH and the degree of hydrolysis when employing the lipolytic enzyme of the invention to paper pulp (determined at 40°C);
Fig. 5 shows three primers (#3831, 17 mer PCR primer + handle, deg. 128; #3832, 17 mer PCR primer + handle, deg. 128; and #4009, 31 mer), designed on basis of the N-terminal amino acid sequence of a lipolytic enzyme of the invention; and
Fig. 6 shows the diagram of plasmid pMT1535.

DETAILED DISCLOSURE OF THE INVENTION

The Microorganisms

[0021] The present invention provides a lipolytic enzyme isolated from a biologically pure culture of a strain of Hyphozyma, which has the ability to produce lipase.

[0022] In a more specific aspect, a biologically pure culture of a new species represented by the strain Hyphozyma sp. LF132, CBS 648.91 is provided. The representative isolate of these novel microorganisms, designated Hyphozyma sp. LF132, has been deposited on 12 November 1991, for the purpose of patent procedures according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms, at Centraal Bureau voor Schimmelcultures (CBS), Oosterstraat 1, 3740 AG Baarn, Netherlands, and is given the accession number CBS 648.91.

[0023] In a further specific aspect, a biologically pure culture of a strain of microorganisms being essentially identical with the native Hyphozyma sp. LF132, CBS 648.91, or mutants or variants thereof is provided.

[0024] The microorganism can be cultivated under aerobic conditions in a nutrient medium containing assimilable carbon and nitrogen together with other essential nutrients, the medium being composed in accordance with the principles of the known art.

[0025] Suitable carbon sources are carbohydrates such as sucrose, glucose and starch, or carbohydrate containing materials such as cereal grain, malt, rice and sorghum. The carbohydrate concentration incorporated in the medium may vary widely, e.g. up to 25% and down to 1 - 5%, but usually 8 - 10% will be suitable, the percentages being calculated as equivalents of glucose.

[0026] The nitrogen source in the nutrient medium may be of inorganic and/or organic nature. Suitable inorganic nitrogen sources are nitrates and ammonium salts. Among the organic nitrogen sources quite a number are used regularly in fermentation processes involving the cultivation of microorganisms. Illustrative examples are soybean meal, cotton seed meal, peanut meal, casein, corn, corn steep liquor, yeast extract, urea and albumin. In addition, the nutrient medium should also contain usual trace substances.

[0027] The cultivation is preferably conducted at pH 4-9, which can be obtained by addition of suitable buffers after sterilization of the growth medium. For cultivation in tank fermentors it is necessary to use artificial aeration. The rate of aeration is similar to that used in conventional tank fermentation.

[0028] After fermentation, liquid enzyme concentrates may be produced by removal of coarse material from the broth or, if desired, concentration of the broth by evaporation at low temperature, or by ultrafiltration or reverse osmosis. Finally, preservatives may be added to the concentrate.

[0029] Solid enzyme preparations may be prepared from the purified and/or concentrated broth by precipitation with

salts, such as Na_2SO_4, or water-miscible solvents, such as ethanol or acetone. Removal of the water in the broth by suitable drying methods, such as spray-drying, may also be employed.

The Enzymes

[0030] The novel lipolytic enzymes of the invention can be described by any of the following characteristics.

Structural Properties

[0031] A lipolytic enzyme of the invention comprises one or more of the following partial amino acid sequences: (a) Phe Thr Pro Phe Pro; (b) Thr Gly Ala Asp Pro; (c) Ala Phe Thr Gln Ser; (d) Gln Ala Thr Leu Asp Ala Gly Leu Thr; (e) Gly Ser Gly Ser Lys; (f) Val Pro Val Leu Thr Trp Ser; (g) Thr Trp Ser Gln Gly Gly Leu Ala Ala Gln; (h) Ala Gln Gln Lys Leu Asp Ser Ala Ala Ile Ile Leu; (i) Val Ala Gly Lys Asn Ile Val Thr Gly Pro Lys Gln; (j) Asn Cys Glu Pro Asp Leu Met Pro Tyr Ala Arg Lys Tyr; and (k) Arg Ile Gly Lys Lys Thr Cys Ser Gly Val Ile Thr Gly.

[0032] In a more specific aspect, a lipolytic enzyme of the invention comprises one or more of the following partial amino acid sequences: (a) Phe Thr Pro Phe Pro Thr Gly Ala Asp Pro; (b) Ala Phe Thr Gln Ser Gln Ala Thr Leu Asp Ala Gly Leu Thr; (c) Gly Ser Gly Ser Lys Val Pro Val Leu Thr Trp Ser; (d) Thr Trp Ser Gln Gly Gly Leu Ala Ala Gln Trp Ala Leu Thr; (e) Ala Gln Gln Lys Leu Asp Ser Ala Ala Ile Ile Leu Val Ala Gly Lys Asn Ile Val Thr Gly Pro Lys Gln; and (f) Asn Cys Glu Pro Asp Leu Met Pro Tyr Ala Arg Lys Tyr Arg Ile Gly Lys Lys Thr Cys Ser Gly Val Ile Thr Gly.

[0033] In a further specific aspect, a lipolytic enzyme of the invention has the following N-terminal amino acid sequence: Phe Thr Pro Phe Pro Thr Gly Ala Asp Pro Ala Phe Thr Gln Ser Gln Ala Thr Leu Asp Ala Gly Leu Thr; or a sequence homologue thereto.

[0034] In a further specific aspect, a lipolytic enzyme of the invention comprises the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Xaa Xaa Xaa Xaa Xaa Ser* Gln Gly Gly.

[0035] In a further specific aspect, a lipolytic enzyme of the invention comprises the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Xaa Xaa Ser* Gln Gly Gly.

[0036] In a further specific aspect, a lipolytic enzyme of the invention comprises the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Thr Xaa Ser* Gln Gly Gly.

[0037] In a further specific aspect, a lipolytic enzyme of the invention comprises the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Pro Xcc Xcc Thr Xaa Ser* Gln Gly Gly.

[0038] In a further specific aspect, a lipolytic enzyme of the invention comprises the following partial amino acid sequence: Gly Ser Gly Xaa Xbb Lys Xaa Pro Val Leu Thr Xaa Ser* Gln Gly Gly.

[0039] In a further specific aspect, a lipolytic enzyme of the invention comprises the following partial amino acid sequence: Gly Ser Gly Ser Xbb Lys Val Pro Val Leu Thr Xaa Ser* Gln Gly Gly.

[0040] In the above partial amino acid sequences Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, Xbb represents a deletion or any of the naturally occurring amino acids except Asn, and Xcc represents a hydrophobic amino acid.

[0041] In a further specific aspect, a lipolytic enzyme of the invention has the amino acid sequence disclosed as SEQ ID NO 3 of the attached amino acid sequence listing, or a sequence homologue thereto.

Table of Amino Acids

One-letter symbol		Symbol		Trivial name
A	=	Ala	=	Alanine
C	=	Cys	=	Cysteine
D	=	Asp	=	Aspartic acid
E	=	Glu	=	Glutamic acid
F	=	Phe	=	Phenylalanine
G	=	Gly	=	Glycine
H	=	His	=	Histidine
I	=	Ile	=	Isoleucine
K	=	Lys	=	Lysine
L	=	Leu	=	Leucine
M	=	Met	=	Methionine
N	=	Asn	=	Asparagine
P	=	Pro	=	Proline

(continued)

Table of Amino Acids				
One-letter symbol		Symbol		Trivial name
Q	=	Gln	=	Glutamine
R	=	Arg	=	Arginine
S	=	Ser	=	Serine
T	=	Thr	=	Threonine
V	=	Val	=	Valine
W	=	Trp	=	Tryptophan
Y	=	Tyr	=	Tyrosine
B	=	Asx	=	Asp or Asn
Z	=	Glx	=	Glu or Gln
X	=	Xaa	=	Unknown or "other" amino acid
*	=	deletion or absent amino acid		

[0042] In the present context, the term "hydrophobic amino acid" encompasses a naturally occurring amino acid having nonpolar or hydrophobic side groups, and includes the following seven amino acids: Ala, Val Leu, Ile, Met, Phe, and Trp.

Homology

[0043] In the present context, the term "homologue" is intended to encompass an amino acid sequence which is at least 75%, preferably at least 85%, most preferred at least 90%, homologous to the sequence referred to. The term is intended to include modifications of the amino acid sequence, which may result in a different protein structure and a lipase mutant with different properties than the native enzyme.

Origin

[0044] In a preferred embodiment, the lipolytic enzyme of the invention is derivable from a strain belonging to the genus

Hyphozyma.

[0045] In a more preferred embodiment, the lipolytic enzyme of the invention is derivable from a strain belonging to the species represented by the strain Hyphozyma sp. LF132, CBS 648.91.

[0046] In a yet more preferred embodiment, the lipolytic enzyme of the invention is derivable from the strain Hyphozyma sp. LF132, CBS 648.91, or a mutant or a variant thereof.

Physico-Chemical Properties

[0047] In another preferred embodiment, the lipolytic enzyme of the invention has more than 80% relative activity in the pH range of from 4.0 to 6.0 (when determined at 70°C).

[0048] In yet another preferred embodiment, the lipolytic enzyme of the invention has a molecular weight of approximately 38-40 kD as determined by SDS-PAGE.

[0049] In a further preferred embodiment, the lipolytic enzyme of the invention has an apparent pI of approximately 6.3, determined by isoelectric focusing on LKB Ampholine® PAG plates.

[0050] In a further preferred embodiment, the lipolytic enzyme of the invention is positionally non-specific.

Immunochemical Properties

[0051] The lipolytic enzyme of the invention is immunologically reactive with an antibody raised against a purified lipase derived from the strain Hyphozyma sp. LF132, CBS 648.91, i.e. has immunochemical properties identical or partially identical (i.e. at least partially identical) to those of a lipase derived from the strain Hyphozyma sp. LF132,

CBS 648.91.

[0052] The immunochemical properties can be determined immunologically by cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to Axelsen N.H.; Handbook of Immunoprecipitation-in-Gel Techniques; Blackwell Scientific Publications (1983), chapters 5 and 14. The terms "antigenic identity" and "partial antigenic identity" are described in the same book, Chapters 5, 19 and 20.

Processes for Obtaining the Lipase

[0053] The lipolytic enzyme of the invention is obtainable by cultivation of a microorganism of the invention, preferably the strain Hyphozyma sp. LF132, CBS 648.91, or a mutant or a variant thereof, in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the enzyme by methods known per se.

[0054] The lipolytic enzyme may also be obtained by recombinant DNA-technology by methods known in the art per se, e.g. isolating a DNA fragment encoding the lipolytic enzyme, combining the DNA fragment with appropriate expression signal(s) in an appropriate vector, introducing the vector or parts thereof into an appropriate host either as an autonomously replicating plasmid or integrated into the chromosome, cultivating the host organism under conditions leading to expression of the lipolytic enzyme, and recovering the lipolytic enzyme from the culture medium.

[0055] In a preferred embodiment, the process comprises cultivating a host organism being an Escherichia coli, a member of the genus Bacillus, Streptomyces or Saccharomyces.

[0056] ,In a more specific embodiment, the process comprises cultivating a host organism being a filamentous fungus, preferably a member of the genus Aspergillus.

[0057] In a yet more specific embodiment, the process comprises cultivating a host organism being A. orvzae or A. niger.

[0058] In another embodiment, the process comprises isolating a DNA fragment having the nucleotide sequence disclosed as SEQ ID NO 2 of the attached sequence listing, or a sequence homologue thereto.

Immobilization of the Lipase

[0059] Immobilized lipase denotes lipase in the form of immobilized enzyme or immobilized cells, as defined in "Guidelines for the characterization of immobilized biocatalysts" (1983), Enzyme Microb. Technol., **5** 304-397.

[0060] For the practice of this invention, the lipolytic enzyme may be immobilized by any method known in the art, e.g. as described in Mosbach K (ed.): Methods in Enzymology, **44**, "Immobilized Enzymes" (Academic Press, 1976). Available methods for enzyme immobilization include cross-linking of cell homogenates, covalent coupling to insoluble inorganic or organic carriers, entrapment in gels, and adsorption on ionexchange resins or other adsorbent materials. Also, coating on a particulate support may be used, as described in Macrae A R and Hammond R C (1985), Biotechnology and Genetic Engineering Reviews, **3** 193.

[0061] A preferred immobilization method uses a particulate, macroporous resin. The lipolytic enzyme may be simply adsorbed on the resin, or it may be attached to the resin by cross-linking with glutaraldehyde or other cross-linking agents known in the art.

[0062] A preferred resin type is weakly basic anion exchange resin, e.g. acrylic, polystyrene or phenolformaldehyde. Another preferred resin type is an adsorbent resin of the phenolformaldehyde type. Yet another preferred resin type is adsorbent resin, e.g. a porous aliphatic olefinic polymer, or of an acrylic type.

[0063] Another preferred immobilization method uses an inorganic support material, and the lipolytic enzyme is preferably attached to the support by adsorption or covalent coupling. Such support materials and immobilization techniques are described in Mosbach K, op. cit.

[0064] In yet another preferred immobilization method, the lipolytic enzyme is immobilized on inorganic materials by adsorption, covalent coupling or precipitation, preferably on zeolites, celites, porous glass beads, glass wool, aluminium oxides, kieselguhr, selicagel, or clay.

[0065] In a further preferred immobilization method, the lipolytic enzyme is immobilized on particles of naturally occurring organic materials, preferably bone particles, chitin, chitosan, or agar.

Enzymatic Pitch Control

[0066] The invention also relates to the use of a lipolytic enzyme of the invention in a method for enzymatic pitch control.

[0067] In the context of this invention, a method for enzymatic pitch control is meant to indicate a method for avoiding pitch troubles that arise in production processes for mechanical pulp or paper-making processes using mechanical pulp. Methods for enzymatic pitch control involve hydrolysis of water-insoluble esters or resins present in the paper pulp.

[0068] A method for enzymatic pitch control may be conducted essentially as described in e.g. International Patent Publications WO 92/07138, WO 92/13130, WO 92/18638, and WO 92/19808.

[0069] In a more specific embodiment, a lipase dosage of 0.5-150 KLU/kg pulp, preferably 20-75 KLU/kg pulp, most preferred 5-20 KLU/kg pulp (dry substance) is used.

[0070] In another specific embodiment, the method is conducted at pH 3-7, preferably 4-7, at a temperature of 40-90°C, preferably 50-70°C, at a reaction time of 0.5-5.0 hours, preferably 2.5-4 hours, and a pulp consistency of 2-30%, preferably 3-8% (w/w).

Lipase-catalyzed Processes

[0071] Due to its excellent thermal stability, the lipolytic enzyme of the invention is advantageously employed in processes performed at elevated temperatures, e.g. synthesis/hydrolysis reactions involving lipids. Moreover, the lipolytic enzyme of the invention is a highly efficient catalyst due to high conversion and low by-product formation.

[0072] The lipolytic enzyme of the invention may be used in any of the following lipase-catalysed processes (reactants indicated in parenthesis):

A) Ester hydrolysis (ester + water)
B) Ester synthesis (acid + alcohol)
C) Interesterification, including:

i) Acidolysis (ester + acid)
ii) Alcoholysis (ester + alcohol)
iii) Ester interchange or transesterification (ester + ester)

[0073] The alcohol may be mono- or polyvalent primary and/or secondary alcohol or a mixture of these. The acid may be any carboxylic acid or a mixture of these. The ester may be any ester derived from the mentioned alcohols and acids, or a mixture of these.

[0074] Some advantageous process embodiments are described in e.g. International Patent Publication WO 88/02775.

[0075] In another preferred embodiment, the lipolytic enzyme of the invention may be used for enzymatic preparation of monoesters of glycosides as described in e.g. US Patent Nos. 5,191,071 and 5,200,328.

Other Industrial Applications

[0076] Among other industrial applications the lipolytic enzyme of the invention may be used for enzymatic manufacture of leather by method known in the art, in order to improve the degreasing of hides and skins, to reduce the use of emulsifiers, and as a substitute for solvents.

[0077] The lipolytic enzyme of the invention can be added either in the soaking, liming or bating, preferably as early in the beamhouse processes as possible. This allows the enzyme sufficient time to operate.

[0078] The following examples further illustrate the present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Cultivation Example

[0079] The strain Hyphozyma sp. LF132, CBS 648.91, was cultivated in a nutrient medium containing the following components (per litre):

Glucose	20 g
Peptone	10 g
$MgSO_4, 7H_2O$	1 g
Yeast Extract	10 g
K_2HPO_4	5 g
pH adjusted to 6.5 with NaOH	

[0080] The strain was cultivated at 30°C for 3 days. The culture broth was subjected to liquid/solid separation by

centrifugation, and the supernatant was freeze-dried resulting in a crude powder preparation.

Lipase Activity

[0081] After centrifugation, a lipase activity of 2 units/g culture broth was obtained, 1 unit being equivalent to the amount of lipase that releases one µmol of fatty acid per minute from emulsified olive oil at 40°C and pH 4.5. The amount of released fatty acid is determined by TLC-FID analysis (Iatroscan™).

Characterization

[0082] The crude powder preparation was characterized by its pH and temperature profile.

[0083] The temperature profile was determined at pH 6.0 in a range of from 40°C to 80°C. The lipase was incubated for 10 minutes, and the activity was determined by the method described above.

[0084] The temperature profile is presented in Fig. 1 as relative activity (setting the activity at 70°C equal to 100%). From the figure it appears that the lipase is active at temperatures of from below 40°C to above 80°C. The temperature optimum of this crude lipase preparation lies in the range of from 60°C to 80°C, more specifically around 70°C.

[0085] The pH profile was determined at 70°C. The lipase was incubated for 10 minutes, and the activity was determined by the method described above.

[0086] The pH profile is presented in Fig. 2 as relative activity (setting the activity at pH 6.0 equal to 100%). Due to a change of buffer system (citrate, phosphate buffer), the figure is made up of two curves, one representing the interval of from pH 4.0 to 6.0, inclusive, the other representing the interval of from pH 6.0 to 7.0, inclusive. From the figure it appears that the lipase is active at pH values of from below 4.0 to above 7.0. No significant pH optimum has been determined, although it appears to be around pH 6.0. However, it also appears that the lipase has more than 80% relative activity within the interval pH 4.0-6.0, preferably more than 90% relative activity in the interval pH 4.0-6.0, when determined at 70°C.

[0087] The lipase was found to act positionally non-specific.

Partial Purification

[0088] 4.0 g of the above crude powder preparation were dissolved in 50 ml 20 mM phosphate buffer, pH 7.2, containing 0.2 M sodium sulfate. The solution was applied on a phenyl sepharose FF column (Pharmacia® LKB Biotechnology AB).

[0089] Lipase was eluted with 0.25 mM phosphate buffer, desalted by ultrafiltration, and freeze dried.

[0090] 300 mg of purified lipase of 15,000 lipase units/g, as described above, were obtained.

EXAMPLE 2

Pulp Treatment

[0091] In this example, the use of a lipase of the invention for triglyceride hydrolysis in paper pulp is demonstrated in laboratory-scale.

[0092] In a first experiment, 500 g of 4% pulp slurry pH were adjusted with H_2SO_4 to 6.5 and 4.5, respectively. 1.5 ml solution containing 100 units of the partially purified lipase obtained according to Example 1 were added. The pulp slurry was incubated for 2 hours at 40, 60, 70, and 80°C, respectively, with stirring (300 rpm.).

[0093] In a second experiment, 500 g of 4% pulp slurry pH were adjusted with H_2SO_4 to 4.0, 4.5, 5.0, 6.0, and 7.0, respectively. 1.5 ml solution containing 100 units of the partially purified lipase obtained according to Example 1 were added. The pulp slurry was incubated for 2 hours at 40°C with stirring (300 rpm.).

[0094] Fatty material was extracted from the lipase treated slurry, and from an untreated slurry (reference slurry), respectively. To 150 g of pulp slurry 150 g of water, 200 ml of hexane and 2 ml of internal standard (1% acetyl cholesterol in hexane) were added. The mixture was shaken for 5 minutes in a separatory funnel, and the pulp filtered off. In the separatory funnel fatty material was collected in the hexane layer, and obtained by evaporation and redissolution.

[0095] Extract was applied on Chromatorod S-III (Iatron Laboratories Inc.), and developed with hexane:ether:NH_4OH (60:8:0.2) mixture. The components were detected by FID analysis (Iatroscan™). The degree of hydrolysis of triglycerides was determined by the calculation:

$$\text{Degree of Hydrolysis} = \frac{TG_0 - TG_R}{TG_0}$$

TG_0: Amount of triglyceride in reference slurry.
TG_R: Amount of triglyceride in lipase-treated slurry.

[0096] The results of the these experiments are presented in Figs. 3-4. It appears from the figures that the lipase of the invention possesses excellent triglyceride hydrolysis in paper pulp in a broad pH range of from pH below 4.0 to pH above 7.0, and in a temperature range of from below 50 to above 70°C.

EXAMPLE 3

Purification Example

[0097] In this example, the lipase activity is described by terms of Lipase Units (LU). One LU is the amount of enzyme which, under standard conditions (i.e. at 30.0°C; pH 7.0; and tributyrine as substrate) liberates 1 µmol of titratable butyric acid per minute. A folder AF 95/5 describing this analytical method is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

[0098] Partially purified, freeze dried lipase powder obtained according to Example 1 (140 to 160 LU/g) was applied on a Butyl-Toyopearl column after adjusting the salt concentration to 0.8 M with ammonium acetate. Bound lipase activity was eluted with water.

[0099] Fractions containing lipase activity were pooled, concentrated and dialysed against 25 mM Tris acetate buffer, pH 7. The concentrated preparation was passed through a DEAE-sepharose column. Effluent containing lipase activity was adjusted to pH 6 and passed through a CM-sepharose column. In both steps, negative adsorption was used to split off impurities.

[0100] Finally, pH of the effluent from the CM-sepharose column was adjusted to 9 and ionic strength to 2 mS/cm and applied on 1 ml of Mono-Q column. The bound lipase activity was eluted with a linear salt gradient. The lipase activity was eluted at or around a salt concentration of 0.15 M.

[0101] Electrophoresis on SDS-PAGE revealed a major band at 38-40 kD (Pharmacia™ Phast method).

EXAMPLE 4

Amino Acid Determination

[0102] The LF132 lipase obtained according to Example 3 was concentrated using a Millipore Ultrafree-MC filter unit. Concomitantly the buffer was changed to 50 mM NH_4HCO_3.

[0103] Following concentration, the sample was subjected to N-terminal amino acid sequence determination in an Applied Biosystems 473A sequencer.

[0104] This determination resulted in the amino acid sequence identified by the sequence listing attached to this specification (SEQ ID NO 1).

EXAMPLE 5

Immobilization Example

[0105] 100 mg of (dry substance) Accurel™ EP100 (which is a particulate polypropylene resin as described in AKZO, Fibres and Polymers Division, Accurel Systems Data Sheet, obtainable from ENKA AG, Postfach D-8753 Obernburg, Germany, were slurried in 96% ethanol. The excess of ethanol was sucked away.

[0106] Immediately 2 ml of an enzyme solution containing the purified lipase obtained according to Example 3 dissolved in phosphate buffer, 50 mM/l, pH 6.5, in an amount of 600 LU/ml, were added. The suspension was stirred for 2 hours at room temperature.

[0107] Subsequently, the product was filtered and rinsed with deionized water (10 ml), and dried in a hood.

EXAMPLE 6

Esterification Example

General Methods

[0108] HPLC-analysis was performed using a Shimadzu LC-4 liquid chromatograph equipped with a RID-2A refractive index detector. A SiO_2-NH_2 Hibar LiChrosorb column (Merck) was used, with 96% ethanol as eluent (Merck, HPLC-

grade). TLC-analysis was performed using SiO_2-coated aluminum sheets (Merck) and toluene/ethylacetate/methanol; 8:6:3 (vol/vol/vol) as mobile phase followed by developing by spraying with 2% sulfuric acid and heating to 100°C. As reference was used ethyl 6-O-dodecanoyl D-glucopyranoside prepared according to Björkling F., Godtfredsen S.E., and Kirk O. (1989); J. Chem. Soc., Chem. Comm. **14** 934-935. ^{1}H-NMR spectra were obtained on a Bruker acp 300 NMR spectrometer using $CDCl_3$ as solvent (using TMS as reference).

Preparation of Ethyl D-glucopyranoside

[0109] D-(+)-Glucose (500 g, 2.8 mol) were suspended in absolute ethanol (1.5 L, 25.7 mol). Amberlyst 15 (strongly acidic ion exchange resin, 20 g) were added and the reaction mixture was refluxed under efficient mechanical stirring. After 16 h HPLC-analysis indicated complete conversion of glucose. The slightly yellow reaction mixture was cooled to room temperature and the ion exchange resin removed by filtration. The crude product was decolorized using activated carbon (5 g), and excess ethanol was distilled off under reduced pressure yielding the crude ethyl D-glucopyranoside as a viscous syrup (^{1}H-NMR indicating a 1:1 mixture of the α- and the β-anomer).

Esterification of Decanol with Dodecanoic Acid

[0110] 150 mg of dodecanoic acid (0.75 mmol) were added to 150 µl of decanol (0.82 mmol), and the mixture was melted at 60°C under magnetic stirring. Then, 10 mg of immobilized lipase (prepared as described in Example 5) were added and stirring was continued at 60°C. After 24 hours the ester formation was monitored by analyzing a sample using ^{1}H NMR. This indicated a conversion of 75% (monitored by comparing the integral of the signals corresponding to the O-CH_2-R group of the ester at 4.05 ppm and the HO-CH_2-R group of the unconverted alcohol at 3.63 ppm).

Esterification of Ethyl D-glucopyranoside with Dodecanoic Acid

[0111] 4 g of ethyl D-glucopyranoside (20 mmol) were mixed with dodecanoic acid (4 g, 40 mmol) at 70°C using mechanical stirring (125 rpm). Then, 400 mg immobilized lipase (prepared as described in Example 5) were added and stirring was continued at 70°C at 0.01 bar. The progress of the reaction was followed by HPLC as described above. After 24 hours a conversion of 88% was reached with a byproduct formation (ethyl 2,6-O-dodecanoyl D-glucopyranoside) of 6%. After 48 hours a conversion of 97% was reached with a by-product formation of 9%.

[0112] This example demonstrates the lipase to be a highly efficient catalyst (high conversion, low by-product formation) in the synthesis of 6-O-monoesters of ethyl D-glucopyranoside, a property which is, certainly, not general for lipases as illustrated in several publications (vide e.g. Björkling F. Godtftedsen S E. and Kirk O, J. Chem. Soc. Chem. Commun. 1989 **14** 934; and Adelhorst K. Björkling F. Godtfredsen S E, and Kirk O, Synthesis, **1990** (2) 111).

EXAMPLE 7

Recombinantly Produced Lipase

[0113] Based on the N-terminal amino acid sequence disclosed in Example 4 two PRC primers (#3831 and #3832, cf. Fig. 5) were designed.

[0114] Using standard techniques (as described in e.g. Sambrook, Fritsch and Maniatis (Eds.), Molecular Cloning, 2. Ed., Cold Spring Harbor Press, 1989), DNA isolated from the strain Hyphozyma sp. LF132, CBS 648.91 was used as template in PCR reactions for amplification of a sequence consistent with the N-terminal sequence. This sequence was cloned as a BamH1-EcoR1 fragment into pUC19 (Yanish-Perron, et al., Gene 1985 **33** 103-109). Sequencing this insert in individual E. coli transformants as expected showed a degenerate sequence in the areas corresponding to the above primers, while the sequence in between was invariant. A primer (#4009, cf. Fig. 5) corresponding to the invariant sequence was synthesized.

[0115] A Sau3A DNA library of the LF132 lipase (4-10 kb) in BamH1-Bgl2 digested pIC19H (Marsh, et al., Gene, 1984 **32** 481-485) was made. The library was probed with primer #4009, and 5 colonies were characterized and shown by restriction mapping to be overlapping clones. The orientation of the clone was determined by running PCR reactions on the clones with primer #4009 and either of the pUC uni or reverse primers (from New England Biolabs, catalogue Nos. 1212 and 1201, respectively). In summary, the entire gene has been localized and subcloned on an approximately 2.6 kb fragment in which the position of the 4009 primer is at approximately 0.5 kb (pMT1535, cf. Fig. 6).

[0116] The part of the pMT1535 insert containing the lipase encoding sequences was subjected to dideoxy sequencing of both strands (cf. SEQ ID NO 2). The N-terminal amino acid sequence of the mature lipase determined in Example 4 was found to be fully in accordance with the sequence starting with phenylalanine in position 23 of the deduced primary translation product.

[0117] The deduced mature protein consists of 319 amino acids with a calculated molecular weight of 33451 D (cf. SEQ ID NO 3).

[0118] Primers #4328 (CGGGATCCTGCAACATGAAGCTCTCG) and #4329 (CGGGATCCTCATCCAGTGATGACGC) were used to introduce BamH1 sit 5' and 3' to the lipase encoding sequence in a PCR product from the above pMT1535. The lipase sequence was confirmed in the pUC19 cloned PCR product. The BamH1-BamH1 fragment was cloned into the vector part of a pMHan37 plasmid, obtained as follows.

[0119] The p960 plasmid, described in EP Patent Application 305,216 and used for expression of Humicola lanuginosa lipase, was modified by replacing 60 basepairs of the 5' untranslated region of the Aspergillus oryzae TAKA promotor just upstream to the Humicola lanuginosa lipase encoding gene with the corresponding 5' untranslated region from the Aspergillus nidulans TPI (triosephosphate isomerase) gene. A synthetic oligonucleotide containing the 5' untranslated region from A. nidulans TPI (triosephosphate isomerase) gene, flanked at each end by 20 bases homologous to p960 sequences just outside the untranslated region, was used in a PCR reaction together with another primer covering the BssHII site in the TAKA promotor region. As the mutagenization primer covers the BamH1 site close to the ATG start codon, the PCR fragment was digested with BamH1 and BssHII, and recloned into p960 digested with BssHII and partially with BamH1, to give the above pMHan37 plasmid.

[0120] The BamH1-BamH1 fragment derived from pMT1535 as described above was cloned into the BamH1 cut and dephosphorylated vector pMHan37. The orientation of the insert was checked by restriction mapping, and one plasmid, pMT1562, in which the LF132 lipase sequence was oriented so as to be under the control of the fungamyl promoter in the expression cassette [Fungamyl promotor - TPI 5' untranslated - preproLF1321ipase - AMG terminator] was obtained.

[0121] pMT1562 was cotransformed into A. oryzae NIBHT 4177 with the selective plasmid pToC90 (obtained according to International Patent Application WO 91/17243). Fifteen transformants were grown in tubes on YP+2% maltose for four days at 30°C, and the supernatants analyzed by SDS gels and coomassie brilliant blue staining. A standard of LF132 lipase, obtained according to Example 3, was run on the same gel.

[0122] Eleven of the transformants appeared to be cotransformants, and from comparison to the standard the best transformants were estimated to make approximately 380 mg/litre when cultivated in shake tubes.

EXAMPLE 8

Thermal Stability

[0123] A lipase preparation obtained according to Example 7 was subjected to analysis for thermal stability by Differential Scanning Calorimetry (DSC). Using this technique, the thermal denaturation temperature, T_d, is determined by heating the enzyme solution at a constant rate and measuring the change in heat capacity during the denaturation process.

[0124] The equipment used was a MC-2D from MicroCal Inc. connected to a PC. Enzyme solutions were prepared in 50 mM degassed buffer (acetate pH 5; TRIS pH 7-9; and glycine pH 10). Enzyme concentration was approx. 0.8 mg/ml as determined by absorbance at 280 nm, and a total volume of 1.2 ml was used. All samples were scanned from 25°C to 90°C at a rate of 90 K/hour.

[0125] The results are presented in Table 1, below. Data for a lipase derived from Candida antarctica (Lipase B, obtained according to International Patent Application WO 88/02775) are shown for comparison, and it is seen that the lipase of the invention is surprisingly more thermostable in defiance of their homology.

Table 1

Thermal Denaturation Temperatures, T_d		
pH	Hyphozyma lipase	Candida B lipase
5	72.4	62.6
7	68.1	62.0
9	60.6	55.1
10	54.2	52.6

SEQUENCE LISTING

[0126]

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma
(B) STRAIN: LF132
(C) INDIVIDUAL ISOLATE: CBS 648.91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Phe Thr Pro Phe Pro Thr Gly Ala Asp Pro Ala Phe Thr Gln Ser Gln
1               5                  10                  15

Ala Thr Leu Asp Ala Gly Leu Thr
            20
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1026 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma
(B) STRAIN: LF132
(C) INDIVIDUAL ISOLATE: CBS 648.91

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 67..1023

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
ATGAAGCTCT CGTCGGCACT TGCCGGTCTG CTGGCCGTCG CCGCAGTTAC TGCCCTCCCT      60

GCCCCC TTT ACA CCC TTC CCC ACG GGC GCA GAC CCG GCC TTC ACT CAA         108
       Phe Thr Pro Phe Pro Thr Gly Ala Asp Pro Ala Phe Thr Gln
         1               5                  10

TCT CAG GCC ACT CTC GAT GCC GGC CTC ACC TGT CAG TCT GGC TCG CCT        156
Ser Gln Ala Thr Leu Asp Ala Gly Leu Thr Cys Gln Ser Gly Ser Pro
 15                  20                  25                  30

TCG TCC CAG AAG AAC CCC ATC CTC CTC GTC CCG GGC ACC GGC AAC ACT        204
Ser Ser Gln Lys Asn Pro Ile Leu Leu Val Pro Gly Thr Gly Asn Thr
                 35                  40                  45

GGC CCA CAG TCG TTC GAC TCG AAC TGG ATT CCG CTT TCC GCC CAG CTC        252
Gly Pro Gln Ser Phe Asp Ser Asn Trp Ile Pro Leu Ser Ala Gln Leu
             50                  55                  60

GGC TAC AGC CCT TGC TGG GTC TCT CCT CCG CCG TTC ATG CTC AAC GAC        300
Gly Tyr Ser Pro Cys Trp Val Ser Pro Pro Pro Phe Met Leu Asn Asp
         65                  70                  75

TCC CAG ATC AAC GCC GAG TAC ATT GTC AAT GCC ATC CAC ACC CTC TCC        348
Ser Gln Ile Asn Ala Glu Tyr Ile Val Asn Ala Ile His Thr Leu Ser
     80                  85                  90

TCG GGC TCC GGG TCC AAG GTT CCT GTT CTG ACC TGG AGT CAA GGT GGT        396
Ser Gly Ser Gly Ser Lys Val Pro Val Leu Thr Trp Ser Gln Gly Gly
 95                  100                 105                 110

CTG GCG GCG CAA TGG GCG CTC ACT TTT TTC CCT AGC ACG CGC AAC AAG        444
Leu Ala Ala Gln Trp Ala Leu Thr Phe Phe Pro Ser Thr Arg Asn Lys
                115                 120                 125

GTC GAC CGC CTG ATG GCT TTT GCT CCT GAC TAC AAG GGC ACC GTT GAA        492
Val Asp Arg Leu Met Ala Phe Ala Pro Asp Tyr Lys Gly Thr Val Glu
            130                 135                 140

GCT GGT CTC CTC GAT GCG TTC GGC CTC AGC GCC CCG AGT GTC TGG CAG        540
Ala Gly Leu Leu Asp Ala Phe Gly Leu Ser Ala Pro Ser Val Trp Gln
        145                 150                 155

CAG ACC GCG CAG TCT GCC TTT GTC ACC GCG CTC GAC CAG GCC GGC GGA        588
Gln Thr Ala Gln Ser Ala Phe Val Thr Ala Leu Asp Gln Ala Gly Gly
    160                 165                 170

TTG AAC CAG ATC GTC CCC ACC ACC AAC CTC TAC TCG GCA ACC GAC GAG        636
Leu Asn Gln Ile Val Pro Thr Thr Asn Leu Tyr Ser Ala Thr Asp Glu
175                 180                 185                 190

GTC GTG CAG CCG CAG TTC GCC AAC GGG CCC CCG GAC TCT TCC TAC CTC        684
Val Val Gln Pro Gln Phe Ala Asn Gly Pro Pro Asp Ser Ser Tyr Leu
                195                 200                 205
```

```
TCT AAC GGC AAG AAC ATC CAG GCA CAG TCG ATC TGC GGC CCG CTC TTC      732
Ser Asn Gly Lys Asn Ile Gln Ala Gln Ser Ile Cys Gly Pro Leu Phe
            210                 215                 220

ATC ATC GGA CAC GCT GGT TCC CTG TAC TCG CAG TTC TCT TAC GTC GTC      780
Ile Ile Gly His Ala Gly Ser Leu Tyr Ser Gln Phe Ser Tyr Val Val
        225                 230                 235

GGC AAG AGT GCG CTC GCC TCG CCC ACC GGT CAG GCC CAG AGC AGC GAT      828
Gly Lys Ser Ala Leu Ala Ser Pro Thr Gly Gln Ala Gln Ser Ser Asp
    240                 245                 250

TAC AGC ATC AAG GAC TGC AAC CCG GCC CCT GCT AAC CCC CTC ACC GCC      876
Tyr Ser Ile Lys Asp Cys Asn Pro Ala Pro Ala Asn Pro Leu Thr Ala
255                 260                 265                 270

CAG CAG AAG CTC GAC TCT GCG GCG ATC ATC CTC GTC GCC GGC AAG AAT      924
Gln Gln Lys Leu Asp Ser Ala Ala Ile Ile Leu Val Ala Gly Lys Asn
                275                 280                 285

ATT GTC ACC GGT CCC AAG CAG AAC TGC GAA CCT GAC CTC ATG CCC TAC      972
Ile Val Thr Gly Pro Lys Gln Asn Cys Glu Pro Asp Leu Met Pro Tyr
            290                 295                 300

GCT CGC AAG TAC CGC ATC GGC AAG AAG ACC TGC TCG GGC GTC ATC ACT     1020
Ala Arg Lys Tyr Arg Ile Gly Lys Lys Thr Cys Ser Gly Val Ile Thr
        305                 310                 315

GGA TGA                                                             1026
Gly
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 319 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Phe Thr Pro Phe Pro Thr Gly Ala Asp Pro Ala Phe Thr Gln Ser Gln
 1               5                  10                  15

Ala Thr Leu Asp Ala Gly Leu Thr Cys Gln Ser Gly Ser Pro Ser Ser
            20                  25                  30

Gln Lys Asn Pro Ile Leu Leu Val Pro Gly Thr Gly Asn Thr Gly Pro
        35                  40                  45

Gln Ser Phe Asp Ser Asn Trp Ile Pro Leu Ser Ala Gln Leu Gly Tyr
    50                  55                  60
```

```
Ser Pro Cys Trp Val Ser Pro Pro Pro Phe Met Leu Asn Asp Ser Gln
 65                  70                  75                  80

Ile Asn Ala Glu Tyr Ile Val Asn Ala Ile His Thr Leu Ser Ser Gly
                 85                  90                  95

Ser Gly Ser Lys Val Pro Val Leu Thr Trp Ser Gln Gly Gly Leu Ala
            100                 105                 110

Ala Gln Trp Ala Leu Thr Phe Phe Pro Ser Thr Arg Asn Lys Val Asp
        115                 120                 125

Arg Leu Met Ala Phe Ala Pro Asp Tyr Lys Gly Thr Val Glu Ala Gly
    130                 135                 140

Leu Leu Asp Ala Phe Gly Leu Ser Ala Pro Ser Val Trp Gln Gln Thr
145                 150                 155                 160

Ala Gln Ser Ala Phe Val Thr Ala Leu Asp Gln Ala Gly Gly Leu Asn
                165                 170                 175

Gln Ile Val Pro Thr Thr Asn Leu Tyr Ser Ala Thr Asp Glu Val Val
            180                 185                 190

Gln Pro Gln Phe Ala Asn Gly Pro Pro Asp Ser Ser Tyr Leu Ser Asn
        195                 200                 205

Gly Lys Asn Ile Gln Ala Gln Ser Ile Cys Gly Pro Leu Phe Ile Ile
    210                 215                 220

Gly His Ala Gly Ser Leu Tyr Ser Gln Phe Ser Tyr Val Val Gly Lys
225                 230                 235                 240

Ser Ala Leu Ala Ser Pro Thr Gly Gln Ala Gln Ser Ser Asp Tyr Ser
                245                 250                 255

Ile Lys Asp Cys Asn Pro Ala Pro Ala Asn Pro Leu Thr Ala Gln Gln
            260                 265                 270

Lys Leu Asp Ser Ala Ala Ile Ile Leu Val Ala Gly Lys Asn Ile Val
        275                 280                 285

Thr Gly Pro Lys Gln Asn Cys Glu Pro Asp Leu Met Pro Tyr Ala Arg
    290                 295                 300

Lys Tyr Arg Ile Gly Lys Lys Thr Cys Ser Gly Val Ile Thr Gly
305                 310                 315
```

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 2, line 17 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (including postal code and country) [5]

Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Netherland

Date of deposit [6]	Accession Number [7]
12 November 1991	CBS 648.91

B. ADDITIONAL INDICATIONS [8] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [9] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [10] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [11] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [12]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

Claims

1. A lipolytic enzyme comprising the following partial amino acid sequence:

Gly Ser Gly Xaa Xbb Lys Xaa Xaa Xaa Xaa Xaa Xaa Ser* Gln Gly Gly;

in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

2. A lipolytic enzyme according to claim 1 comprising the following partial amino acid sequence:

Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Xaa Xaa Ser* Gln Gly Gly;

in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

3. A lipolytic enzyme according to claim 2 comprising the following partial amino acid sequence:

Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Thr Xaa Ser* Gln Gly Gly;

in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

4. A lipolytic enzyme according to claim 3 comprising the following partial amino acid sequence:

Gly Ser Gly Xaa Xbb Lys Xaa Pro Xcc Xcc Thr Xaa Ser* Gln Gly Gly;

in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, Xbb represents a deletion or any of the naturally occurring amino acids except Asn, and Xcc represents a hydrophobic amino acid.

5. A lipolytic enzyme according to claim 4 comprising the following partial amino acid sequence:

Gly Ser Gly Xaa Xbb Lys Xaa Pro Val Leu Thr Xaa Ser* Gln Gly Gly;

in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

6. A lipolytic enzyme according to claim 5 comprising the following partial amino acid sequence:

Gly Ser Gly Ser Xbb Lys Val Pro Val Leu Thr Xaa Ser* Gln Gly Gly;

in which sequence Ser* represents the catalytically active serine, Xaa represents any of the naturally occurring amino acids, and Xbb represents a deletion or any of the naturally occurring amino acids except Asn.

7. A lipolytic enzyme according to claim 1 having the amino acid sequence of SEQ ID NO: 3.

8. A process for obtaining a lipolytic enzyme according to claims 1-7, which process comprises cultivation of a lipase producing strain of the genus *Hyphozyma* in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the lipolytic enzyme.

9. A method for enzymatic pitch control comprising addition of a lipolytic enzyme according to claim 1-7, for hydrolysis of water-insoluble esters.

10. A method according to claim 9, in which a lipase dosage of 0.5-150 KLU/kg pulp, (dry substance) is employed.

11. A method according to claim 9-10, conducted at pH 3-7, at a temperature of 40-90°C, at a reaction time of 0.5-5.0 hours, and a pulp consistency of 2-30% (w/w).

12. An immobilized lipase preparation obtainable by immobilization of the lipolytic enzyme of claims 1-7.

13. An immobilized lipase preparation according to claim 12, wherein the lipase is immobilized on a particulate, macroporous weakly basic anion exchange resin.

14. An immobilized lipase preparation according to claim 12, wherein the lipase is immobilized on a particulate, porous non-ionic adsorbent resin, preferably a porous aliphatic olefinic polymer or of an acrylic type.

15. An immobilized lipase preparation according to claim 12, wherein the lipase is immobilized on inorganic materials by adsorption, covalent coupling or precipitation.

16. An immobilized lipase preparation according to claim 12, wherein the lipase is immobilized on particles of naturally occurring organic materials.

Patentansprüche

1. Lipolytisches Enzym, umfassend die folgende partielle Aminosäuresequenz:

Gly Ser Gly Xaa Xbb Lys Xaa Xaa Xaa Xaa Xaa Xaa Ser* Gln Gly Gly,

in welcher Sequenz Ser* das katalytisch aktive Serin darstellt, Xaa irgendeine der natürlich vorkommenden Aminosäuren darstellt und Xbb eine Deletion oder irgendeine der natürlich vorkommenden Aminosäuren mit Ausnahme von Asn darstellt.

2. Lipolytisches Enzym nach Anspruch 1, umfassend die folgende partielle Aminosäuresequenz:

Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Xaa Xaa Ser* Gln Gly Gly,

in welcher Sequenz Ser* das katalytisch aktive Serin darstellt, Xaa irgendeine der natürlich vorkommenden Aminosäuren darstellt und Xbb eine Deletion oder irgendeine der natürlich vorkommenden Aminosäuren mit Ausnahme von Asn darstellt.

3. Lipolytisches Enzym nach Anspruch 2, umfassend die folgende partielle Aminosäuresequenz:

Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Thr Xaa Ser* Gln Gly Gly,

in welcher Sequenz Ser* das katalytisch aktive Serin darstellt, Xaa irgendeine der natürlich vorkommenden Aminosäuren darstellt und Xbb eine Deletion oder irgendeine der natürlich vorkommenden Aminosäuren mit Ausnahme von Asn darstellt.

4. Lipolytisches Enzym nach Anspruch 3, umfassend die folgende partielle Aminosäuresequenz:

Gly Ser Gly Xaa Xbb Lys Xaa Pro Xcc Xcc Thr Xaa Ser* Gln Gly Gly,

in welcher Sequenz Ser* das katalytisch aktive Serin darstellt, Xaa irgendeine der natürlich vorkommenden Aminosäuren darstellt, Xbb eine Deletion oder irgendeine der natürlich vorkommenden Aminosäuren mit Ausnahme von Asn darstellt und Xcc eine hydrophobe Aminosäure darstellt.

5. Lipolytisches Enzym nach Anspruch 4, umfassend die folgende partielle Aminosäuresequenz:

Gly Ser Gly Xaa Xbb Lys Xaa Pro Val Leu Thr Xaa Ser* Gln Gly Gly,

in welcher Sequenz Ser* das katalytisch aktive Serin darstellt, Xaa irgendeine der natürlich vorkommenden Aminosäuren darstellt und Xbb eine Deletion oder irgendeine der natürlich vorkommenden Aminosäuren mit Ausnahme von Asn darstellt.

6. Lipolytisches Enzym nach Anspruch 5, umfassend die folgende partielle Aminosäuresequenz:

Gly Ser Gly Ser Xbb Lys Val Pro Val Leu Thr Xaa Ser* Gln Gly Gly,

in welcher Sequenz Ser* das katalytisch aktive Serin darstellt, Xaa irgendeine der natürlich vorkommenden Aminosäuren darstellt und Xbb eine Deletion oder irgendeine der natürlich vorkommenden Aminosäuren mit Ausnahme von Asn darstellt.

7. Lipolytisches Enzym nach Anspruch 1 mit der Aminosäuresequenz von SEQ-ID-NR. 3.

8. Verfahren zur Gewinnung eines lipolytischen Enzyms nach den Ansprüchen 1-7, wobei das Verfahren die Kultivierung eines Lipaseproduzierenden Stammes der Gattung *Hyphozyma* in einem geeigneten Nährmedium, das Kohlenstoff- und Stickstoffquellen und anorganische Salze enthält, gefolgt von Gewinnung des lipolytischen Enzyms, umfaßt.

9. Verfahren zur enzymatischen Zellstoffharzbekämpfung, umfassend die Zugabe eines lipolytischen Enzyms nach Anspruch 1-7 zur Hydrolyse von wasserunlöslichen Estern.

10. Verfahren nach Anspruch 9, worin eine Lipase-Dosierung von 0,5-150 KLE/kg Zellstoff (Trockensubstanz) eingesetzt wird.

11. Verfahren nach Anspruch 9-10, welches bei pH 3-7, bei einer Temperatur von 40-90° C, bei einer Reaktionszeit von 0,5-5,0 Stunden und einer Zellstoffkonsistenz von 2-30 % (Gew./Gew.) durchgeführt wird.

12. Immobilisierte Lipase-Präparation, erhältlich durch Immobilisierung des lipolytischen Enzyms nach den Ansprüchen 1-7.

13. Immobilisierte Lipase-Präparation nach Anspruch 12, worin die Lipase auf einem aus Teilchen bestehenden, makroporösen, schwach basischen Anionenaustauschharz immobilisiert ist.

14. Immobilisierte Lipase-Präparation nach Anspruch 12, worin die Lipase auf einem aus Teilchen bestehenden, porösen, nicht-ionischen, adsorbierenden Harz, vorzugsweise einem porösen, aliphatischen, olefinischen Polymer oder einem vom Acryl-Typ, immobilisiert ist.

15. Immobilisierte Lipase-Präparation nach Anspruch 12, worin die Lipase auf anorganischen Materialien durch Adsorption, kovalente Kopplung oder Präzipitation immobilisiert ist.

16. Immobilisierte Lipase-Präparation nach Anspruch 12, worin die Lipase auf Teilchen von natürlich vorkommenden organischen Materialien immobilisiert ist.

Revendications

1. Une enzyme lipolytique comprenant la séquence partielle d'acides aminés suivante :

Gly Ser Gly Xaa Xbb Lys Xaa Xaa Xaa Xaa Xaa Xaa Ser* Gln Gly Gly,

où la séquence Ser* représente la sérine catalytiquement active, Xaa représente l'un quelconque des acides aminés présents dans la nature et Xbb représente une délétion ou l'un quelconque des acides aminés présents dans la nature à l'exception d'Asn.

2. Une enzyme lipolytique selon la revendication 1, comprenant la séquence partielle d'acides aminés suivante :

Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Xaa Xaa Ser* Gln Gly Gly,

où la séquence Ser* représente la sérine catalytiquement active, Xaa représente l'un quelconque des acides aminés présents dans la nature et Xbb représente une délétion ou l'un quelconque des acides aminés présents dans la nature à l'exception d'Asn.

3. Une enzyme lipolytique selon la revendication 2, comprenant la séquence partielle d'acides aminés suivante :

Gly Ser Gly Xaa Xbb Lys Xaa Pro Xaa Xaa Thr Xaa Ser* Gln Gly Gly,

où la séquence Ser* représente la sérine catalytiquement active, Xaa représente l'un quelconque des acides aminés présents dans la nature et Xbb représente une délétion ou l'un quelconque des acides aminés présents dans la nature à l'exception d'Asn.

4. Une enzyme lipolytique selon la revendication 3, comprenant la séquence partielle d'acides aminés suivante :

Gly Ser Gly Xaa Xbb Lys Xaa Pro Xcc Xcc Thr Xaa Ser* Gln Gly Gly,

où la séquence Ser* représente la sérine catalytiquement active, Xaa représente l'un quelconque des acides aminés présents dans la nature, Xbb représente une délétion ou l'un quelconque des acides aminés présents dans la nature à l'exception d'Asn et Xcc représente un acide aminé hydrophobe.

5. Une enzyme lipolytique selon la revendication 4, comprenant la séquence partielle d'acides aminés suivante :

Gly Ser Gly Xaa Xbb Lys Xaa Pro Val Leu Thr Xaa Ser* Gln Gly Gly,

où la séquence Ser* représente la sérine catalytiquement active, Xaa représente l'un quelconque des acides aminés présents dans la nature et Xbb représente une délétion ou l'un quelconque des acides aminés présents dans la nature à l'exception d'Asn.

6. Une enzyme lipolytique selon la revendication 5, comprenant la séquence partielle d'acides aminés suivante :

Gly Ser Gly Ser Xbb Lys Val Pro Val Leu Thr Xaa Ser* Gln Gly Gly,

où la séquence Ser* représente la sérine catalytiquement active, Xaa représente l'un quelconque des acides aminés présents dans la nature et Xbb représente une délétion ou l'un quelconque des acides aminés présents dans la nature à l'exception d'Asn.

7. une enzyme lipolytique selon la revendication 1, comportant la séquence d'acides aminés de SEQ ID NO: 3.

8. Un processus d'obtention d'une enzyme lipolytique selon les revendications 1 à 7, processus qui comprend la culture d'une souche productrice de lipase du genre *Hyphozyma* dans un milieu nutritif approprié, contenant des sources de carbone et d'azote et des sels inorganiques, suivie de la récupération de l'enzyme lipolytique.

9. Un procédé de contrôle de l'indice enzymatique, comprenant l'addition d'une enzyme lipolytique selon les revendications 1 à 7, pour l'hydrolyse d'esters insolubles dans l'eau.

10. Un procédé selon la revendication 9, dans lequel on emploie un dosage en lipase de 0,65 à 150 KLU/kg de pulpe (matière sèche).

11. Un procédé selon les revendications 9 à 10, conduit à pH 3 à 7, à une température de 40 à 90°C, avec un temps de réaction de 0,5 à 5,0 heures et une consistance de pulpe de 2 à 30 % (poids/poids).

12. Une préparation de lipase immobilisée susceptible d'être obtenue par immobilisation de l'enzyme lipolytique des revendications 1 à 7.

13. Une préparation de lipase immobilisée selon la revendication 12, où la lipase est immobilisée sur une résine échangeuse d'anions particulaire macroporeuse faiblement basique.

14. Une préparation de lipase immobilisée selon la revendication 12, où la lipase est immobilisée sur une résine adsorbante non ionique particulaire poreuse, de préférence un polymère oléfinique aliphatique ou d'un type acrylique.

15. Une préparation de lipase immobilisée selon la revendication 12, où la lipase est immobilisée sur des matériaux inorganiques par adsorption, couplage covalent ou précipitation.

16. Une préparation de lipase immobilisée selon la revendication 12, où la lipase est immobilisée sur des particules de matériaux organiques présents dans la nature.

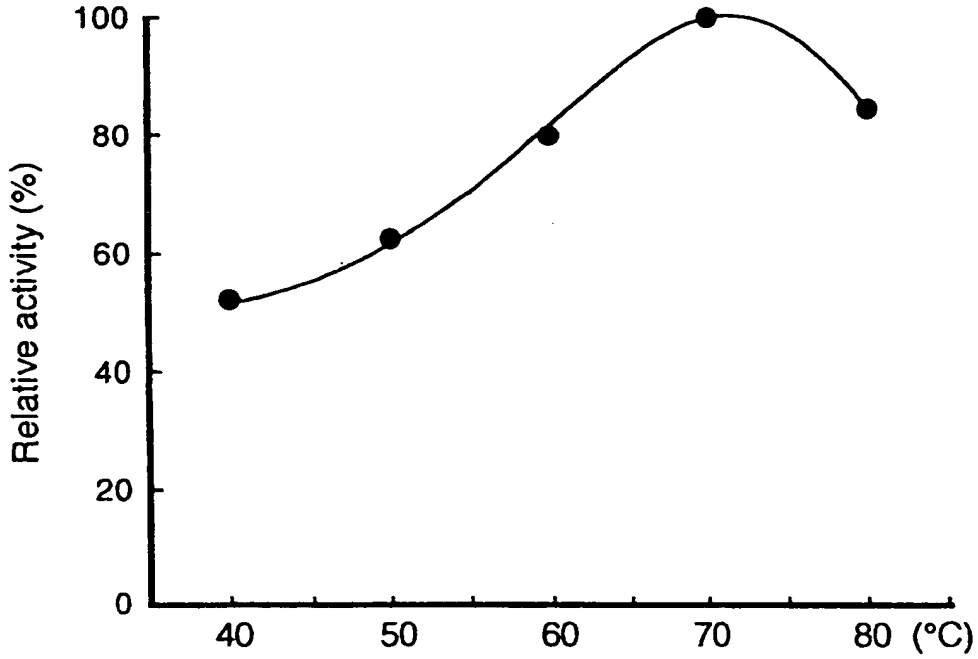

Fig. 1

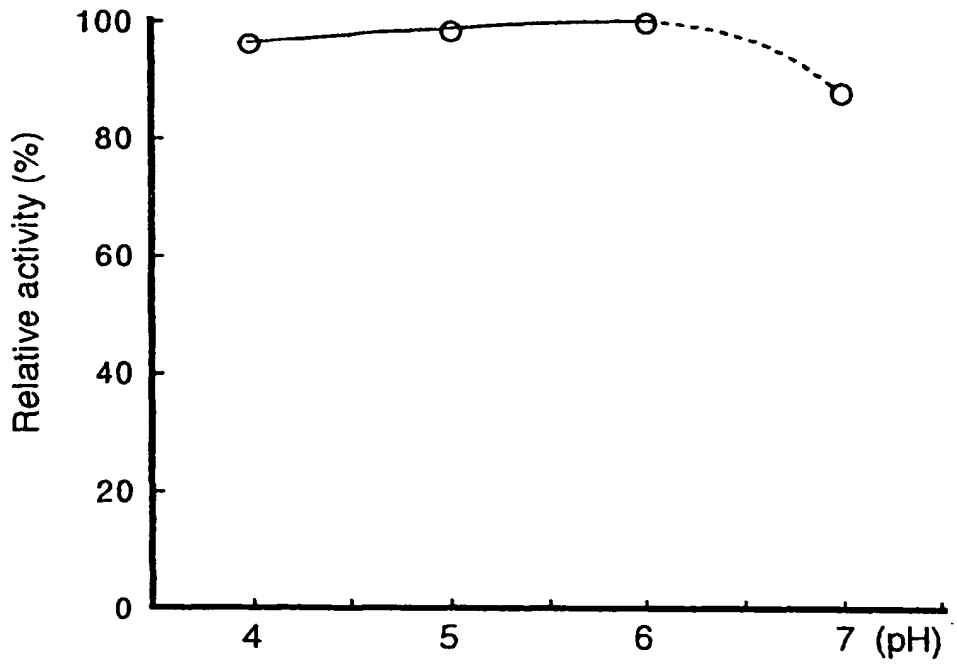

Fig. 2

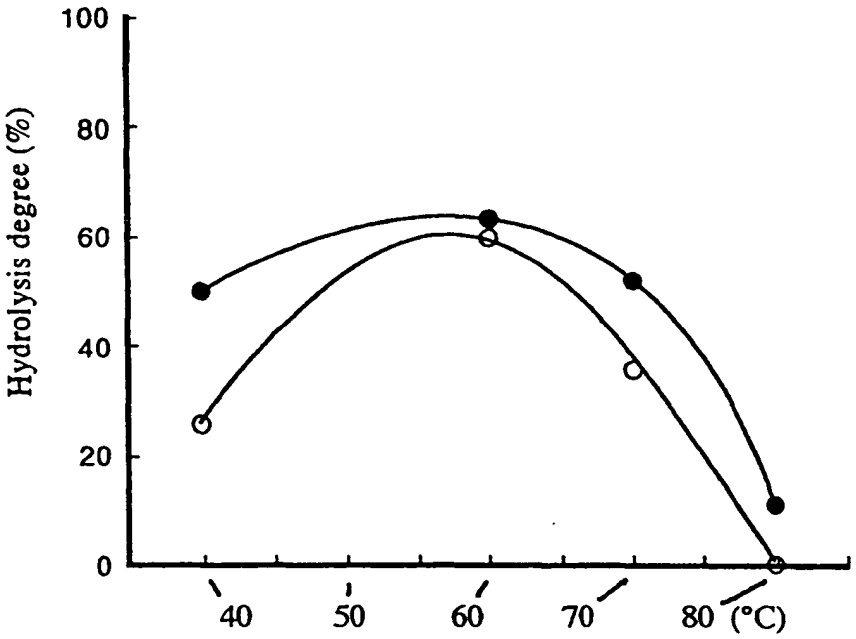

Fig. 3

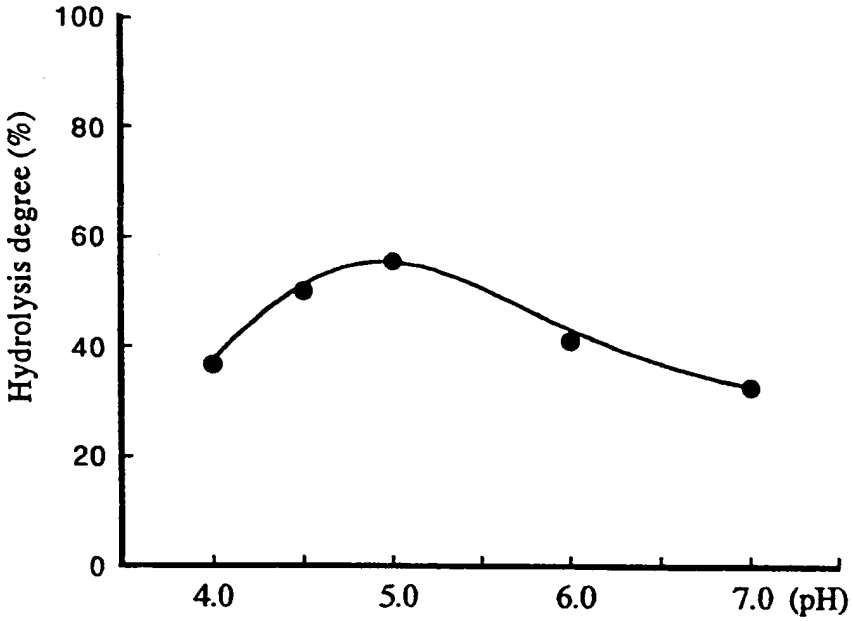

Fig. 4

LF 132 N-terminal

```
        F  T  P  F  P  T  G  A  D  P  A  F  T  Q  S  Q  A  T  L  D  A

        TTCACACCATTCCCAACAGGAGCAGACCCAGCATTCACACAATCACAAGCAACACTAGACGCA
          T  C  C  T  C  C  C  C  T  C  C  T  C  G  C  G  C  C  C  T  C
             G  G     G  G  G  G     G  G     G     G     G  G  G     G
             T  T     T  T  T  T     T  T     T     T     T  T  T     T
                                                 AGC         TTA
                                                   T           T
```

```
CGGAATTCTTCACACCATTCCCAAC
            C  C  T  C
            G  G     G
            T  T     T
```

3831
17 mer PCR primer+
handle
deg: 128

3832
17 mer PCR primer +
handle
deg:128

```
3' GTCCGATGAGAACTGCGCCTAGGGC
      T  C  C  C
         G  G  G
         T  T  T
```

Primer 4009 ACGGGCGCAGACCCGGCCTTCACTCAATCTC
31 mer

Fig. 5

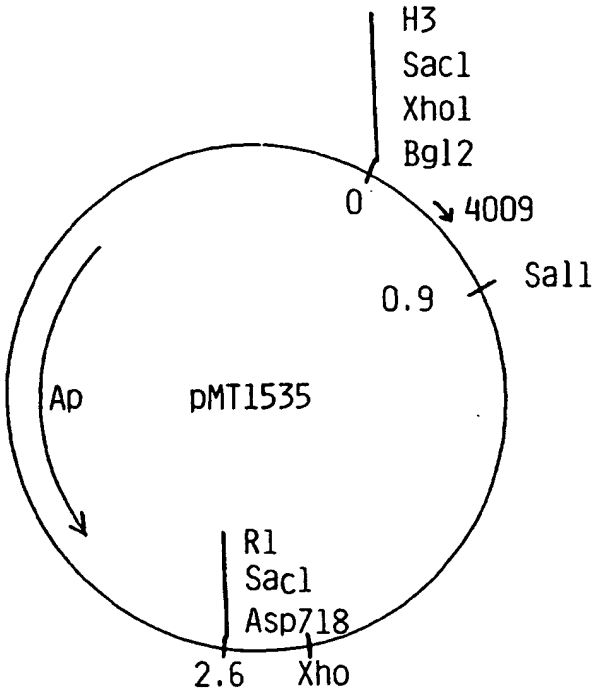
H3
SacI
XhoI
BglI2
0
4009
SalI
0.9
Ap
pMT1535
RI
SacI
Asp718
2.6
Xho

Fig. 6